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Award Number: DAMD17-00-1-0567

TITLE: Role of oocyte loss in ovarian surface mesothelial cell transformation

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REPORT DATE: November 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2002	Annual (1 Oct 01 - 1 Oct 02)

4. TITLE AND SUBTITLE

Role of oocyte loss in ovarian surface mesothelial cell transformation

5. FUNDING NUMBER

DAMD17-00-1-0567

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Three *Specific Aims* (SA) were proposed to test in mice the hypothesis that accelerated oocyte loss caused by Bclw deficiency or Bax gain-of-function drives ovarian surface mesothelial cell (OSMC) transformation: 1) characterize preneoplastic changes in OSMC of *bclw*$^{-/-}$ mice with increasing age; 2) determine if disruption of the gene encoding Bax, a Bclw interacting partner required for oocyte apoptosis, rescues the compromised oocyte survival and the OSMC transformation phenotype observed in aging *bclw*$^{-/-}$ mice; and, 3) test if targeting overexpression of *bax* to *only* growing oocytes accelerates oocyte depletion and causes OSMC transformation. To date, we have generated the mice needed to complete SA 1 and 2, and have confirmed the occurrence of OSMC transformation in 9+ month *bclw* mutants (SA 1). However, there is no evidence of progression to invasive carcinoma by 20+ months of age (SA 1). We have confirmed that simultaneous inactivation of *bax* restores the compromised oocyte endowment in *bclw* mutants to normal (SA 2). Finally, we have constructed the *zp3-bax* minigene and have begun to generate transgenic mice expressing Bax only in growing oocytes (SA 3), and have shown in another mouse model that accelerated oocyte loss is directly involved in ovarian tumorigenesis.

14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)	15. NUMBER OF PAGES
ovarian surface mesothelium, apoptosis, transformation, oocytes	10
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT Unlimited
Unclassified	Unclassified	Unclassified	

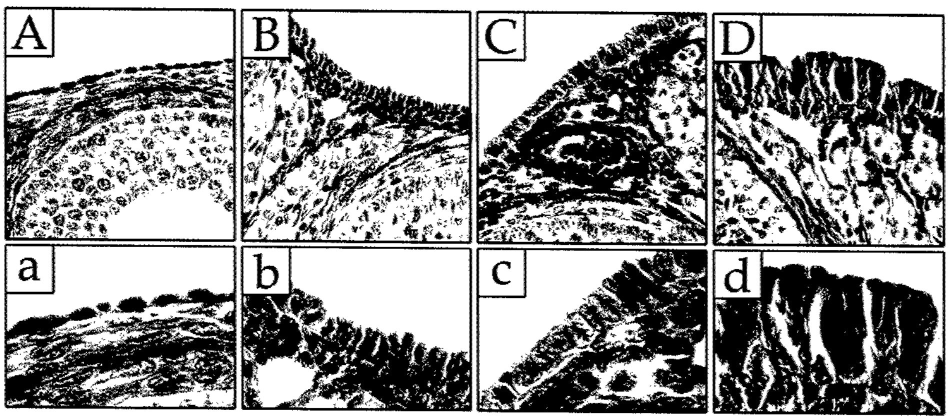
Table of Contents

INTRODUCTION: Ovarian cancer accounts for approximately 4% of the total cancers diagnosed in humans per year, and is the seventh most common cause of tumors in women. The majority of patients diagnosed with ovarian cancer are between 50-60 years of age, and 60-70% of these women present with advanced stages of the disease (stage III or IV) at the time of diagnosis. It is widely accepted that the vast majority (85-90% or more) of ovarian cancers in humans originate from the ovarian surface mesothelium (OSM). However the mechanisms underlying the onset of the disease, as well as the early pre-neoplastic changes that occur prior to invasive (stage I) ovarian carcinoma, remain a mystery. In the original application for this *Idea Award*, the investigators provided evidence supporting a novel etiology for OSM cell transformation, that being accelerated depletion of the female germ cell (oocyte) population. Preliminary studies in mice revealed that targeted disruption of the gene encoding Bclw, an anti-apoptotic member of the Bcl2 family of programmed cell death regulators, leads to premature loss of the oocyte pool followed by pre-neoplastic transformation of OSM cells around mid-life (similar to humans). The process appears to phenotypically copy many of the pre-cancerous cellular changes observed in other gynecologic epithelial lineages (*e.g.*, cervical and uterine tissues) prior to the development of stage I carcinoma. Therefore, the following *Specific Aims* were proposed to test the hypothesis that accelerated oocyte loss drives transformation of OSM cells: 1) analyze the pre-neoplastic changes in OSM cells of $bclw^{-/-}$ female mice with increasing post-natal age using histopathology and immunohistochemical screening of known antigens expressed by normal versus transformed human OSM cells; 2) determine if disruption of the gene encoding Bax, a Bclw interacting partner required for oocyte apoptosis to proceed, rescues the compromised oocyte survival and the OSM cell transformation phenotype observed in aging $bclw^{-/-}$ female mice; and, 3) test if targeting over-expression of the *bax* cell death-promoting gene to *only* growing oocytes of post-natal female mice leads to accelerated oocyte depletion followed by OSM cell transformation.

BODY: Over the past year (Year 2 of the grant), we have focused our efforts on two principal areas, both of which we feel are critical to establishing the validity of our central hypothesis that accelerated oocyte depletion, *per se*, is a driving force behind the development of ovarian cancer. The first area was the generation of the transgenic mice outlined in Specific Aim 3 of the proposal, in which the promoter of the oocyte-specific gene, *zona pellucida protein-3* (*zp3*), was to be used to drive expression of the pro-apoptotic *bax* gene as a way to 'cleanly' accelerate oocyte depletion. In Year 1 of the grant, we successfully constructed the minigene necessary to generate these mice, and in Year 2 were performed our first series of pronuclear injections to produce founder mice. The second area was a complementary approach based on preliminary and published information presented as a rationale for this work in the original proposal. Specifically, we utilized the fact that irradiation of young adult female mice accelerates oocyte loss, leading to premature ovarian failure and – of direct relevance to our central hypothesis – ovarian cancer later in life. To test if this latter outcome is associated with the oocyte loss that accompanies radiation treatment of females, we conducted a series of experiments in which ovaries of female mice were pretreated with vehicle or a lipid mediator of oocyte survival, sphingosine-1-phosphate (S1P), prior to irradiation The mice were then housed long-term and evaluated for the incidence of tumor development. The results generated thus far from these experiments, as well as other studies related to the completion of this *Idea Award*, are detailed below, followed by a brief summary of the projected time frame for completion of the work.

For *Specific Aim 1* (analyze the pre-neoplastic changes in OSM cells of *bclw*$^{-/-}$ female mice with increasing post-natal age), we have generated all of the mice needed to investigate changes in the ovaries of *bclw* mutant females during post-natal life into advanced chronological age (20+ months). We have confirmed the occurrence of the pre-cancerous OSM cell transformation in the *bclw*-null females at 9+ months of age (Figure 1), and have found by histological and histochemical approaches that the phenotype does not progress into invasive carcinoma by 20+ months of age (data not shown). Therefore, we must conclude that the accelerated oocyte depletion caused by *bclw* gene disruption in female mice facilitates hyperplastic growth and transformation of the OSM cells without progression to invasive carcinoma. However, given the multifactorial/multistep nature of tumor development, in retrospect such a finding may not be surprising. Accordingly, the generation of mice simultaneously lacking the tumor suppressor protein, p53, in the context of accelerated oocyte depletion (see *Specific Aim 3* below) may provide the 'genetic' environment needed to allow the hyperplastic OSM cells, produced as a consequence of oocyte loss, to commit to carcinoma development.

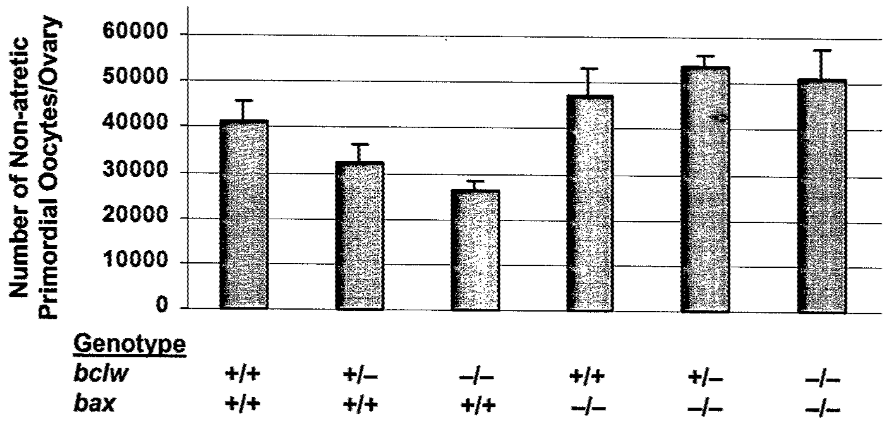
FIGURE 1. Histological analysis of the ovaries of wild-type (A, a) and *bclw*-null (B-D, b-d) female mice at 9-9.5 months of age. Note the dramatic change in the morphology of the OSM cell population from a squamous (wild-type) to a columnar (mutants) phenotype. Furthermore, note the increase in the nuclear:cytoplasmic ratio in, and the dysplastic aggregation of, the OSM cells of *bclw* mutant mice. Panels designated by lower case letters are higher magnifications of the images shown in the corresponding panels designated by capital letters.



For *Specific Aim 2* (determine if disruption of the gene encoding Bax, a Bclw interacting partner required for oocyte apoptosis to proceed, rescues the compromised oocyte survival and the OSM cell transformation phenotype observed in aging *bclw*$^{-/-}$ female mice), we have generated all of the mice needed to investigate changes in the ovaries of *bclw*/*bax* double-mutant females, along with wild-type and single gene mutant controls, during post-natal life into advanced chronological age (16+ months). We have completed the histomorphometric quantitation of oocyte numbers in wild-type, *bclw* mutant/*bax* wild-type, *bax* mutant/*bclw* wild-type, and *bclw*/*bax* double mutant females at day 4 postpartum (Figure 2), and have nearly finished our series of oocyte counts at day 42 postpartum and 9 months of age postpartum (data not shown as the slides are still coded until we complete all of the counts for each age group). As proposed in Specific Aim 2, *bax* gene knockout does in fact prevent the loss of oocytes caused by *Bclw*-

deficiency. However, since these counts are conducted 'blind' (the slides are coded), we will not have the opportunity to perform the histopathological analyses of ovaries collected from the females of the latter age groups for OSM cell transformation until the oocyte counts are completed and the slides have been decoded. This will be completed this year.

FIGURE 2. Quantitative analysis of primordial oocyte endowment in wild-type (*bclw*$^{+/+}$/*bax*$^{+/+}$), *bclw*$^{+/-}$/*bax*$^{+/+}$, *bclw*$^{-/-}$/*bax*$^{+/+}$, *bclw*$^{+/+}$/*bax*$^{-/-}$, *bclw*$^{+/-}$/*bax*$^{-/-}$ and *bclw*$^{-/-}$/*bax*$^{-/-}$ female mice at day 4 postpartum. These data represent the mean±SEM of combined results from analyzing oocyte numbers in a minimum of three mice per genotype.



For *Specific Aim 3* (test if targeting over-expression of the *bax* cell death-promoting gene to only growing oocytes of post-natal female mice leads to accelerated oocyte depletion followed by OSM cell transformation), we have successfully completed construction of the transgene vector containing a fragment of the murine *zona pellucida protein-3* (*zp3*) gene promoter (for oocyte-specific expression) upstream of the cDNA coding sequence of human *bax*. The transgene was grown to large scale, sequenced and restriction enzyme-mapped to confirm its fidelity, and purified for pronuclear injection to generate transgenic mice expressing recombinant human Bax protein only in growing oocytes. The first round of pronuclear injections of the transgene into one-cell embryos was conducted this year, and 8 transgene-positive or founder animals were generated. These animals were allowed to reach adulthood and then tested for germline transmission of the transgene. Unfortunately, none of the 8 founders were capable of germline transmission, necessitating that we repeat the pronuclear injections and produce additional founder animals. At this time, however, Dr. Grant MacGregor, the Co-Principal Investigator who is overseeing this specific aspect of the proposal, accepted a new faculty position at the University of California-Irvine. Dr. MacGregor has since moved from Emory University to UC-Irvine and is in the process of re-assembling his laboratory and research team. This move for Dr. MacGregor, while important for his career goals, produced an unanticipated delay of about 4-6 months for us to complete the generation of the transgenic mice with germline transmission. Although we fully expect that these mice will be generated long before the end of Year 3 (September 30, 2003), we are now unsure if the *aging* studies can be accomplished with these mice in this time frame. Accordingly, we may have to submit a request for a 6-month no-cost extension with the U.S. Army Medical Research and Materiel Command during this final year of

funding so that the histopathology can be conducted on ovaries collected from *zp3-bax* transgenic mice at 9+ months of age.

Also related to *Specific Aim 3*, and as indicated in our annual report covering October 2000-September 2001, we plan to test if simultaneous inactivation of the *p53* gene in these transgenic mice either accelerates the time frame for OSM cell transformation or enables the OSM transformation process to progress to invasive carcinoma. To do this, the resultant transgenic mice, once generated, will be immediately outcrossed with $p53^{-/-}$ mice (Jackson Laboratories, Bar Harbor, ME) to produce *zp3-bax* transgenic/*p53*-null females for parallel analysis with the *zp3-bax* transgenic/p53 wild-type mice. We have already acquired several breeding pairs of heterozygous *p53* animals, and have begun expanding this colony of mice in anticipation of the generation of the transgenic mice. A revised Statement of Work is being prepared for submission to the Contracting Officer Representative (COR) detailing this new objective, which, as pointed out by the Reviewer of our annual report covering October 2000-September 2001, represents a logical extension of the originally proposed research. Moreover, given the findings this year that the aged *bclw*-null females do not show evidence of invasive carcinoma (see *Specific Aim 1* above), we feel that this new experiment is now even more critical to attempt.

In addition, a second new objective is being added to the revised Statement of Work that is directly relevant to the central hypothesis of the proposal. As discussed in the original application as part of the rationale for this work, other (but more noxious) methods of induced oocyte loss in female mice, such as irradiation, lead to early ovarian failure followed by the development of pre-cancerous (tubular mesothelial adenomas) and cancerous (complex mixed cell lineage tumors, often containing granulosa cells) lesions in the ovaries later in life (reviewed in *J Exp Pathol* 1987 3:115-145). While our approach is much 'cleaner' with respect to causing premature oocyte elimination in the absence of collateral damage to other cell types (and thus establishing cause-effect relationships are much easier), the radiation-induced ovarian cancer model is nonetheless intriguing because of its association with accelerated oocyte loss. To determine if the development of ovarian cancer in irradiated female mice is related to the oocyte depletion, we utilized an approach recently validated by our laboratory which protects the female germ line from destruction following exposure to radiation (*Nature Medicine* 2000 6:1109-1114). This essentially entails pretreatment of young adult female mice with a single intrabursal injection of the anti-apoptotic lipid molecule, sphingosine-1-phosphate (S1P), prior to irradiation. The massive oocyte loss observed in vehicle-pretreated irradiated animals does not occur in S1P-pretreated irradiated females, thus providing us with a unique model to explore the contribution of oocyte loss versus global (collateral) radiation-induced ovarian cell damage to the development of ovarian cancer. For these experiments, the mice were treated and irradiated at 2 months of age, and ovaries were then collected at 12 months of age for histopathological analysis. As shown in Table 1, all seven vehicle-pretreated irradiated female mice exhibited nearly complete (n=1) or complete (n=6) ovarian failure – defined by the absence of multi-layer follicles containing viable oocytes. This was correlated with pre-cancerous lesion (tubular mesothelial adenoma) formation in all seven animals and cancerous lesion (complex mixed cell/granulosa cell tumor) formation in five of the seven animals. In striking contrast, only three of the seven S1P-pretreated irradiated females exhibited ovarian failure. And while these three animals showed evidence of pre-cancerous lesion formation, <u>none</u> exhibited tumor development. Furthermore, of the four remaining S1P-pretreated irradiated female mice still retaining multi-

layer follicles (oocytes), only one exhibited evidence of tubular mesothelial adenoma formation, but again none exhibited evidence of tumor development (Table 1). Although additional mice may be needed to increase the sample size before we can draw final conclusions, these exciting new data strongly support the validity of our central hypothesis that accelerated oocyte loss is a critical factor involved in the pathogenesis of ovarian cancer.

TABLE 1. Incidence of ovarian failure, pre-cancerous ovarian lesion (tubular mesothelial adenoma) formation, and ovarian cancer (mixed cell/granulosa cell tumor) development in 12-month old female mice pretreated with vehicle or S1P prior to irradiation at 2 months of age.

Treatment Group	Mouse	Ovarian Failure	**Adenomas**	**Tumors**
No Irradiation	n=7	No (many oocytes)	No	No
Vehicle+Radiation	SII-1	Yes	**Yes**	**Yes**
	SII-2	Yes	**Yes**	No
	SII-3	Yes	**Yes**	**Yes**
	SII-4	No (few oocytes)	**Yes**	**Yes**
	SII-5	Yes	**Yes**	**Yes**
	SII-6	Yes	**Yes**	No
	SII-7	Yes	**Yes**	**Yes**
S1P+Radiation	SII-10	No	No	No
	SII-11	Yes	**Yes**	No
	SII-12	No	**Yes**	No
	SII-13	Yes	**Yes**	No
	SII-14	No	No	No
	SII-15	Yes	**Yes**	No
	SII-16	No	No	No

With respect to our goals during the *final year* of this project (Year 3; October 2002-September 2003), we fully anticipate the completion of Specific Aims 1 and 2, and the submission of these combined data in one manuscript for publication. For Specific Aim 3, we remain convinced that the transgenic mice will be generated (based on the fact that we have already produced 8 founders) and that some will have the capacity for germline transmission of the transgene. However, as indicated above, Dr. MacGregor's unanticipated professional move from Emory University to UC-Irvine has delayed the generation of these mice by 4-6 months. Accordingly, we are unsure if we can complete the *aging* studies (9+ months of age) in these mice by the projected termination date of this award (September 2003). If needed, a 6-month no-cost extension may be requested of the U.S. Army Medical Research and Materiel Command to complete these final experiments. Lastly, we hope to build on the exciting new preliminary data shown in Table 1 that, through a completely different but complementary approach, directly link

oocyte loss to ovarian cancer development, and to finalize these data for publication over the next year as well. Therefore, we believe that at the conclusion of this *Idea Award*, we will have produced unequivocal evidence of a novel role for oocyte loss in ovarian cancer, as well as a number of new and valuable mouse models for exploring the early stages of OSM cell transformation and ovarian cancer development in future investigations.

KEY RESEARCH ACCOMPLISHMENTS (Bulleted List; Changes and Additions from 2000-2001 Annual Report are Highlighted by Underlining):

•Generated all single and double gene mutant female mice needed, at all of the appropriate ages, to satisfy the objectives of Specific Aims 1 and 2

•Collected sera and ovaries from all single and double gene mutant female mice needed, at all of the appropriate ages, to satisfy the objectives of Specific Aims 1 and 2

•Confirmed the occurrence of OSM cell transformation in 9+ month *bclw* mutant females, and found that there is no evidence of progression to invasive carcinoma by <u>20+ months of age by both histological and histochemical criteria</u> (Specific Aim 1)

•Confirmed that simultaneous inactivation of the *bax* gene restores the compromised oocyte endowment in *bclw* mutant females at day 4 postpartum to normal (Specific Aim 2)

•<u>Nearly completed oocyte counts for the latter age groups of single and double mutant mice (Specific Aim 2)</u>

•Completed construction of the *zp3-bax* transgene vector needed for generation of mice with targeted over-expression of the pro-apoptotic Bax protein to only growing oocytes, as needed for Specific Aim 3

•Confirmed fidelity of the *zp3-bax* transgene vector by sequence analysis and restriction enzyme mapping, and purified transgene for pronuclear injections (Specific Aim 3)

•<u>Performed first round of pronuclear injections and generated 8 founder offspring exhibiting incorporation of the transgene in the genome (Specific Aim 3)</u>

•<u>Tested if any of the 8 founder *zp3-bax* transgenic animals were capable of germline transmission of the transgene to offspring (Specific Aim 3)</u>

•<u>Determined that ovarian cancer development in aging female mice resulting from gonadal irradiation in young adult life was related to accelerated oocyte depletion</u>

REPORTABLE OUTCOMES:

Abstracts

•Maravei DV, Ross A, Waymire K, Morita Y, Robles R, Korsmeyer SJ, MacGregor GR, Tilly JL. *Bax* gene inactivation rescues gametogenic failure caused by Bcl-w-deficiency but not *ataxia telangiectasia mutated* (*Atm*) gene knockout. Proceedings of the 82nd Annual Meeting of the Endocrine Society, Toronto, Ontario, Canada 2000; pp 317-318

•Tilly JL. Genes and mechanisms of ovarian failure. Proceedings of the 84th Annual Meeting of the Endocrine Society, San Francisco, CA 2002; p 41

Presentations/Invited Lectures

•NIH Workshop on The Ovary: Genesis, Function and Failure, Bethesda, MD; "Genetics of Programmed Cell Death (PCD) in Normal and Premature Ovarian Failure"; March 2000.

•Department of Cell Biology Distinguished Lecturer Series, University of Medicine and Dentistry of New Jersey, Stratford, NJ; "Use of Gene Knockouts to Navigate the Muddy Waters of Oocyte Apoptosis"; April 2000.

•NICHD-Sponsored 3rd Symposium on Frontiers in Reproduction, The Oocyte and Human Reproduction, Boston, MA; "Prenatal Oocyte Apoptosis: A Genetic Balancing Act"; June 2000.

•Symposium on Oocyte Development, 82nd Annual Meeting of the Endocrine Society, Toronto, Ontario, Canada; "Gene Knockout Analysis of Oocyte Apoptosis During Gametogenesis"; June 2000.

•Gordon Research Conference on Mammalian Gametogenesis and Embryogenesis, New London, CT; "Programmed Cell Death Signaling Pathways in Developing Oocytes"; July 2000.

•18th Annual Meeting of the Japanese Society for Fertilization and Implantation, Okazaki, Japan; "Genetics of Programmed Cell Death in Mammalian Oocytes"; July 2000.

•Institut de Recherches Servier, Suresnes, Paris, France; "Ovarian Failure and Ovarian Cancer: Two Sides of the Same Coin?"; November 2000.

•Annual Meeting of the Triangle Consortium for Reproductive Biology (TCRB), NIEHS, Research Triangle Park, NC; "Mouse and Human Models to Study the Molecular Genetics of Ovarian Cell Death"; January 2001.

•Keystone Symposium on the Molecular Mechanisms of Apoptosis, Keystone, CO; "Apoptosis in Mouse and Human Models of Ovarian Failure"; January 2001.

•Symposium on Ovarian Failure, 84th Annual Meeting of the Endocrine Society, San Francisco, CA; "Genes and Mechanisms of Ovarian Failure"; June 2002.

Animal Models

•Mutant female mice lacking *bclw*

•Double-mutant female mice lacking both *bclw* and *bax*

•Irradiated female mice with S1P-based preservation of the oocyte pool

REFERENCES: no data have been published in *manuscript* form as of yet.

APPENDICES: none.